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## Characterization of Lysine 56 of Histone H3 as an Acetylation Site in *Saccharomyces cerevisiae*\*<sup>§</sup>

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**Post-translational histone modifications abound and regulate multiple nuclear processes. Most modifications are targeted to the amino-terminal domains of histones. Here we report the identification and characterization of acetylation of lysine 56 within the core domain of histone H3. In the crystal structure of the nucleosome, lysine 56 contacts DNA. Phenotypic analysis suggests that lysine 56 is critical for histone function and that it modulates formamide resistance, ultraviolet radiation sensitivity, and sensitivity to hydroxyurea. We show that the acetylated form of histone H3 lysine 56 (H3-K56) is present during interphase, metaphase, and S phase. Finally, reverse genetic analysis indicates that none of the known histone acetyltransferases is solely responsible for H3-K56 acetylation in *Saccharomyces cerevisiae*.**

In eukaryotes, genetic information is packed in a higher order structure of histones and genomic DNA that is called chromatin. The fundamental unit of chromatin is the nucleosome and consists of 147 bp of DNA wrapped about twice around a histone octamer that contain a histone H3/H4 tetramer and two H2A/H2B dimers (1, 2). Post-translational modifications of the histone tails are linked to different states of chromatin that regulate processes like transcription, DNA repair, replication, and recombination (3–5). Overlapping actions of histone modifying enzymes on the very same or different histone residues generates a combinational complexity of modifications that is called the histone code (5). Hyperacetylation of lysines located in the amino-terminal tail of core histones correlates with transcriptional activation whereas hypoacetylation relates to transcriptional repression (3, 4). Histone acetylation is a dynamic process that is regulated by the opposing activities of histone acetyltransferases (HATs)<sup>1</sup> (6) and histone

deacetylases (7). Methylation status of lysines in the amino-terminal tail, and the histone-fold domain of histone H3 plays an important role in the establishment of the active (and/or silenced) state of chromatin (5, 8).

In contrast, not much is known about histone core domain modifications and their functions. Recently, acetylation of histone H4 lysine 91 was shown to be important for chromatin assembly (9). It is also known that methylation of histone H3 lysine 79 impinges on transcription silencing (10, 11). Furthermore, a globular domain histone mutation, H3 leucine 61 to tryptophan, impaired association of SWI/SNF with chromatin (12). Here we identify and characterize acetylation of histone H3 lysine 56 as a novel core domain histone modification in *S. cerevisiae*.

### MATERIALS AND METHODS

**Yeast Strains, Plasmids, and Media**—A list of the strains we employed is provided as supplemental Table 1. Plasmid [pHHT2-HIS3] was made by insertion of a 1010-base pair HindIII-SnaBI DNA fragment excised from [pMR366-URA3-HHT2] (13), encompassing the HHT2 open reading frame plus 408 base pairs upstream and 210 base pairs downstream DNA. Site-directed mutagenesis on [pHHT2-HIS3] was confirmed by sequencing the entire gene. Where indicated, compounds were added to the following final concentrations; 0.2% (w/v) 5-fluoroorotic acid (5-FOA; ICN Biochemicals), 100 mM hydroxyurea (HU; Sigma), 0.01% (v/v) methyl methanesulfonate (Acros Organics), 3% (v/v) formamide (Fluka Biochemica), 15  $\mu$ g/ml nocodazole (Sigma). A Stratagene UV Stratalinker was used to score sensitivity to UV irradiation.

**Antiserum against Acetylated Histone H3 lysine 56 (H3-K56)**—A polyclonal H3-K56[Ac] serum was raised by immunizing a rabbit with the RRFQK[Ac]STELLIRKL synthetic peptide conjugated to keyhole limpet hemocyanin.

**Histone Purification**—Histones were purified according to Edmondson *et al.* (14) except that zymolyase (Seikagaku Corp. catalog no. 120493) was used at a final concentration of 0.1 mg/ml.

**SDS-PAGE and Western Blots**—SDS-PAGE and Western blot analysis were performed according to standard procedures (15). Purified histones were separated on 15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Schleicher & Schuell). Membranes were incubated at 4 °C for 3 h in TBST (20 mM Tris, pH 8.0, 125 mM NaCl, and 0.05% Tween 20) with antibodies either against acetylated histone H3-K56 (1:300 dilution in TBST), diacetyl histone H3 (Upstate Biotechnology catalog number 06-599, 1:1000), acetylated histone H3-K18 (Abcam catalog number ab1191, 1:1000), tetra-acetyl histone H4 (Upstate Biotechnology catalog number 06-866, 1:1000), dimethyl histone H3-K4 (Abcam catalog number ab7766, 1:1000), trimethyl histone H3-K4 (Abcam catalog number ab8580, 1:1000), or against histone H3 (Abcam catalog number ab1791, 1:1000). Western blots were developed with an ECL detection kit (Amersham Biosciences).

**Flow Cytometry Analysis**—Cellular DNA content was determined as described (16) using 1  $\mu$ M sytox green (Molecular Probes) and a BD Biosciences calibur fluorescence activated cell sorter.

**Purification of Active HAT Fractions**—Histone acetyltransferase activity was purified as described previously (17). Whole-cell extract that was prepared from a 10-liter yeast culture was loaded onto Ni<sup>2+</sup>-nitrilotriacetic acid-agarose (Qiagen), eluted with 0.3 M imidazole buffer, and then applied to a Mono Q column (Amersham Biosciences). H3-K56 HAT activity eluted at 200 mM NaCl.

### RESULTS AND DISCUSSION

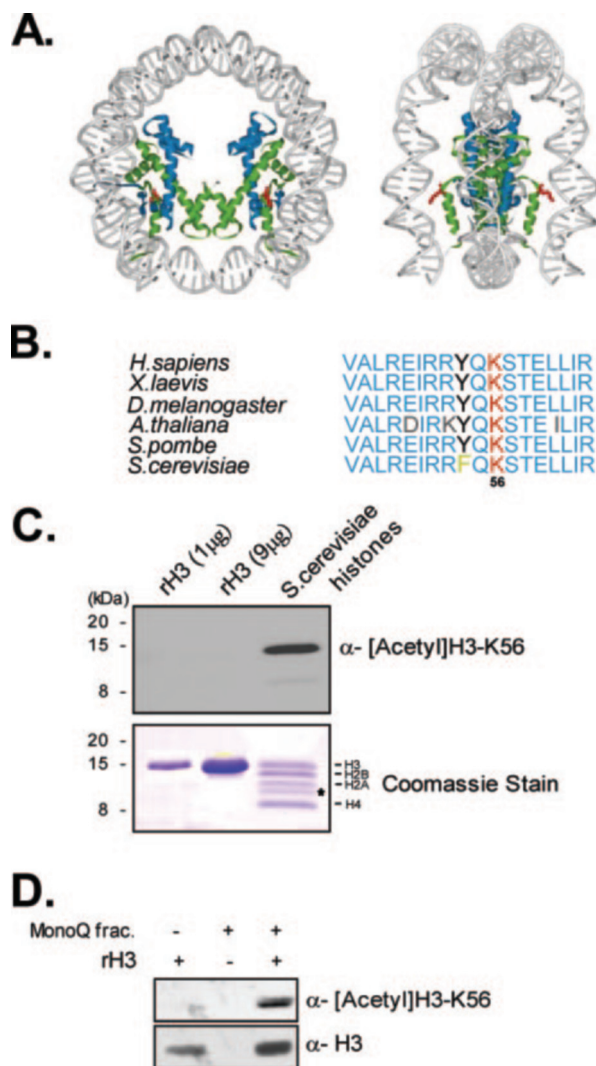
**Identification of a Novel Histone Modification**—Acetylation of H3-K56, a novel core domain histone H3 modification in *S. cerevisiae* was identified multiple times by mass spectrometry analysis of histone preparations (data not shown). Zhang *et al.* (18) did not detect acetylation of histone H3 lysine 56 using calf thymus histones, although evidence for methylation

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§ The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables 1 and 2.

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<sup>1</sup> The abbreviations used are: HAT, histone acetyltransferase; 5-FOA, 5-fluoroorotic acid; HU, hydroxyurea; H3-K56, histone H3 lysine 56; YEPD, yeast extract-peptone-dextrose.



**FIG. 1. Characterization of H3-K56 acetylation.** A, crystal structure of the yeast nucleosome. For simplicity histone H2A/H2B dimers are not depicted. DNA is shown in gray, histone H3 in green, and H4 in blue. H3-K56 is highlighted in red. The structure is based on Protein Data Bank code 1ID3. B, alignment of histone H3 (amino acids 46–63) from different species. C, analysis of recombinant and native yeast histones. In the upper panel, *E. coli* expressed (lanes 1 and 2) and acid-extended yeast (YN1037) histones (lane 3) were analyzed by Western blot using rabbit serum raised against a synthetic acetylated H3-K56 peptide. In the lower panel, Coomassie Blue-stained 15% SDS-polyacrylamide gel shows the quality of histone protein preparations. The protein marked with the asterisk is a proteolytic fragment of histone H3. D, a representative *in vitro* HAT assay is shown; reactions were analyzed by Western blot using an antibody against acetylated H3-K56 (upper panel) or the core domain of H3 (lower panel).

of arginine 52 or 53, or of lysine 56, of histone H3 was obtained. Crystal structure analysis of the nucleosome (1) revealed that H3-K56 is located on a side of the H3/H4 tetramer facing DNA (Fig. 1A). To characterize this new modification, we raised an antibody against a synthetic peptide carrying acetylated H3-K56. The antibody recognizes a protein band that comigrates with purified histone H3 (Fig. 1C, lane 3). It does not recognize recombinant yeast histone H3 expressed in *Escherichia coli* (rH3) (Fig. 1C, lanes 1 and 2). However, the antiserum does recognize rH3 after an *in vitro* HAT reaction using active yeast extract (Fig. 1D, lane 3). Phenylalanine at position 54 of histone H3 is not conserved in other species (Fig. 1B), therefore the antiserum is specific for the acetylated form of *S. cerevisiae* histone H3-K56, and it does not recognize mammalian histone H3 (data not shown).

**Analysis of the Mutant *hht2* Alleles**—To further validate that the antibody specifically recognizes acetylated H3-K56, and to gain insight into the possible function(s) of this modification, we constructed budding yeast strains that expressed wild type and mutant alleles of *HHT2* from low copy number plasmids as sole source of histone H3. The effect on viability of point mutations at position 56 was assayed in a yeast strain, YN1375, lacking both chromosomal copies of H3. This strain harbored wild type *HHT2* on a *URA3* plasmid. Hence, medium containing 5-FOA did not permit growth of YN1375 (Fig. 2A). The single amino acid substitutions of histone H3 lysine 56 to alanine (H3-K56A) or to arginine (H3-K56R) borne by the *HIS3* plasmid sustained viability of YN1375 on 5-FOA plates, indicating functionality (Fig. 2A). In contrast histone H3 bearing a glutamate at position 56 (H3-K56E) could not support cell proliferation (Fig. 2A).

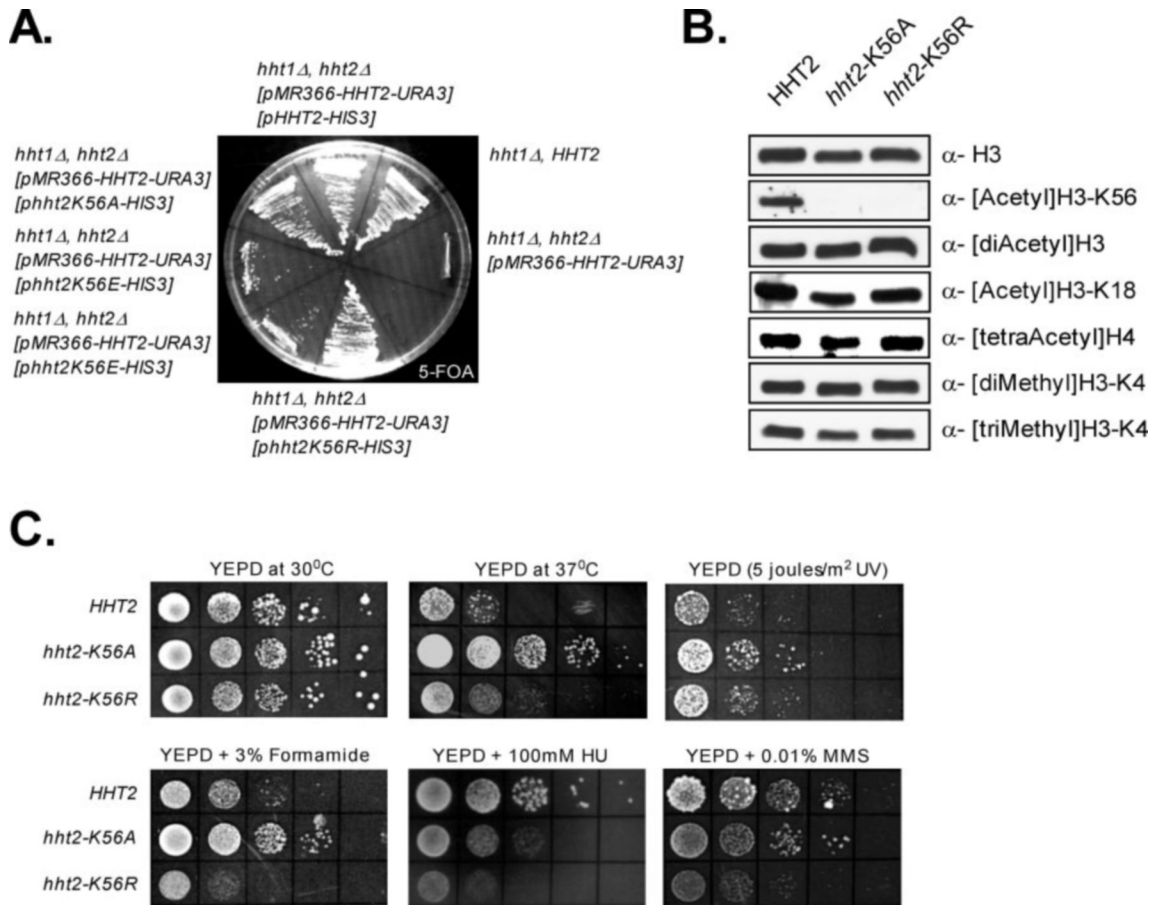
Both H3-K56A and H3-K56R substitutions disrupted the epitope as such that H3-K56 [acetyl] antibody recognition of the mutant H3 histones was abolished (Fig. 2B, panel 2). To exclude the possibility that the level of histone H3 was affected in the mutants we used a commercial antibody that recognizes another epitope within the core domain of histone H3. As shown in Fig. 2B (panel 1), the total amount of histone H3 is similar in all strains. These results indicate that the antiserum we raised is highly specific for acetylated H3-K56.

**Interplay with Other Histone Modifications**—A particular modification that is present on a histone residue may coexist with, or be required for, modifications at other residues (3). Acetylation of lysines that are located at the N-terminal tail of histones H3 and H4 are associated with transcription activation (4, 5). We sought to find out whether the acetylation of H3-K56 was a determinant of known histone tail modifications. To this end, we purified histones from strains expressing H3-K56A (YN1392) or H3-K56R (YN1393) as a sole source of histone H3. Global acetylation levels of histone H3 and histone H4 N-terminal tails were not affected in the *hht2-K56A* and *hht2-K56R* mutants (Fig. 2B, panels 3–5). The levels of di- and trimethylation of histone H3-K4 were not different either (Fig. 2A, panels 6 and 7). These findings suggest that H3-K56 acetylation is not required for the establishment and/or the maintenance of these epigenetic marks at the genome wide level. We note that this does not exclude the possibility that acetylation of H3-K56 might influence the levels of histone modifications at specific loci.

**Phenotype Analysis of the *hht2-K56A* and *hht2-K56R* Alleles**—To better understand the function of H3-K56 acetylation, we performed a phenotypic analysis on the *hht2-K56A* and *hht2-K56R* alleles. Single amino acid substitution of a lysine to an arginine (*hht2-K56R*) is predicted to cause no major changes within the structure of the H3/H4 tetramer. Because of the position of the residue (Fig. 1A); however, we expect to retain ionic interactions between histone H3 and DNA, which would promote a more stable chromatin template. Alanine on the other hand is a smaller amino acid than lysine and is not charged. Therefore substitution to an alanine (*hht2-K56A*) is expected to weaken the interactions between histone H3 and DNA, thereby destabilizing the nucleosome and creating a more flexible environment for chromatin remodelers and transcription associated regulatory protein complexes.

Temperature sensitivity is a common yeast phenotype (19). Surprisingly, the *hht2-K56A* allele conferred a growth advantage to the cells at 37 °C relative to the *HHT2* and the *hht2-K56R* alleles (Fig. 2C, first row). It has been reported that 30% of formamide-sensitive strains also display temperature sensitivity (19). We therefore also tested formamide sensitivity. Not much is known about the molecular mechanisms that underpin



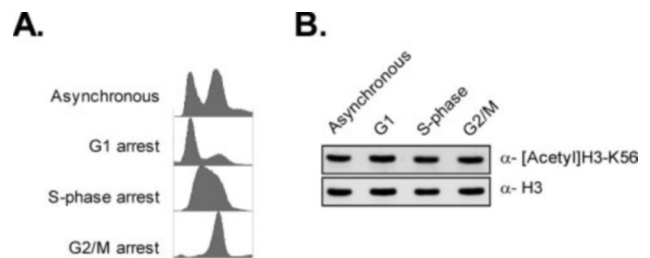


**FIG. 2. Analysis of histone H3 lysine 56 point mutations.** A, the *hht2-K56A* and *hht2-K56R* alleles support viability, *hht2-K56E* does not. B, analysis of other histone H3 modifications in H3-K56 mutants. Histones from the strains carrying either the wild type *HHT2* (YN1391, lane 1), or the mutant *hht2-K56A* (YN1392, lane 2), and *hht2-K56R* (YN1393, lane 3) alleles of histone H3 were analyzed by Western blot using antibodies against a core domain histone H3 (panel 1), acetylated H3-K56 (panel 2), diacetyl histone H3 (panel 3), acetyl H3-K18 (panel 4), tetra-acetyl histone H4 (panel 5) and against di- and trimethyl histone H3-K4 (panels 6 and 7). C, phenotypes conferred by the *hht2-K56A* and *hht2-K56R* alleles. Cells were grown to mid-log phase at 30 °C in selective SD media, and then 10-fold serial dilutions were spotted on the indicated medium. Pictures of plates incubated at 30 °C for 3–4 days are shown.

this phenotype, although it likely reflects hydrogen bridge destabilization. The *hht2-K56A* allele also displayed a growth advantage on YEPD containing 3% formamide (Fig. 2C, second row), whereas yeast strains carrying either the *wild type* or the *hht2-K56R* alleles of histone H3 were clearly defective for growth on this medium. This would suggest that suppression of the lethality induced by formamide is not a result of the loss of acetylation at lysine 56 but that it is associated with a structural advantage conferred by the alanine substitution onto the nucleosome.

HU is an inhibitor of ribonucleotide reductase; hence exposure to HU causes yeast cells to arrest in S phase of the cell cycle. Growth of both mutant strains was clearly retarded on YEPD + HU relative to the wild type strain, and the effect of the *hht2-K56R* allele was much more pronounced (Fig. 2C, second row). The same results were obtained when methyl methanesulfonate was used instead of HU (Refs. 16 and 19; Fig. 2C, second row). This phenotype implies a possible role of H3-K56 acetylation in DNA replication-coupled repair and/or progression through the S phase of the cell cycle.

Sensitivity to UV irradiation indicates defects in DNA damage repair responses. Mutants bearing either *hht2-K56A* or *hht2-K56R* alleles of histone H3 showed a significant increase of survival when exposed to 5 joules/m<sup>2</sup> of UV irradiation (Fig. 2C, first row). We envisage two explanations for this phenotype; either a lethal DNA damage-induced cell cycle block is circumvented, or the repair pathway is constitutively on in the



**FIG. 3. Acetylation of H3-K56 during the cell cycle.** A, flow cytometry analysis. Wild type cells were used for asynchronous cultures (YN1037, panel 1). *cdc25-2* cells were arrested in G<sub>1</sub> by 5 h of heat shock at 37 °C (YN133, panel 2). S phase and G<sub>2</sub>/M arrests were achieved by growing yeast (YN1037) for 4 h in YEPD containing hydroxyurea or nocodazole, respectively (panels 3 and 4). B, histones extracted from cell cycle staged yeast (Fig. 3A) were analyzed by Western blot using anti-serum against acetylated H3-K56 (panel 1). Lanes 1–4 correspond to histones purified from asynchronous, G<sub>1</sub>-, S-phase, and G<sub>2</sub>/M-arrested cells, respectively. Relative amount of the histone H3 in each sample was quantified using an antibody against histone H3 (panel 2).

mutant strains. This could be due to a direct involvement of H3-K56 acetylation in repair process or indirectly via an altered cellular transcription related profile.

**Cell Cycle Regulation of H3-K56 Acetylation**—The fact that the mutant *hht2-K56A* and *hht2-K56R* alleles of histone H3 showed DNA damage repair and replication-related phenotypes may be taken to indicate that H3-K56 acetylation takes

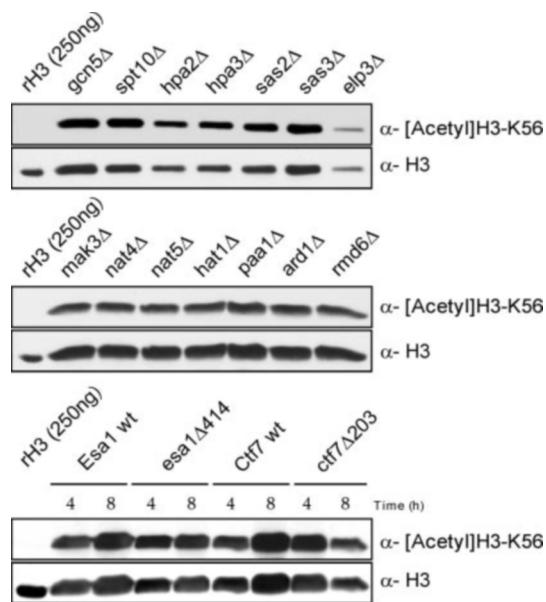


FIG. 4. Screen to identify the HAT responsible for acetylation of H3-K56 (see supplemental Table 1 for strains). Genetic deletion mutants for non-essential HATs (supplemental Table 2) were grown to saturation at 30 °C in YEPD. Cells carrying either wild type or temperature-sensitive (*ts*) alleles of the essential *Esa1* and *Ctf7* acetyltransferases were grown to mid-log phase at 25 °C, then shifted to 37 °C. Samples were collected after 4 and 8 h of heat shock. Histones were extracted from all samples and analyzed for the presence of the modification by Western blot.

place at a defined stage of the cell cycle. To examine this possibility, we assayed for the presence of H3-K56 acetylation in  $G_1$ -, S-, and  $G_2$ /M phase-arrested *S. cerevisiae* (Fig. 3A). This revealed that H3-K56 acetylation is present in  $G_1$ , S-phase, and  $G_2$ /M (Fig. 3B).

**Screen for the H3-K56 Acetyltransferase**—To identify the HAT responsible for this novel histone modification, we performed a screen with deletion strains of the major putative HATs (supplemental Table 1). There are two classes of HATs: the A-type HATs are located in the nucleus and acetylate nucleosomal histones, and the B-type HATs on the other hand are located in the cytoplasm and acetylate free histones (6). Because H3-K56 is likely to bind DNA (Fig. 1A), we expect that the acetylation occurs on free histone H3. To be accurate, however, we included both classes in our experiments (supplemental Table 2). *Esa1p* and *Ctf7p* are essential acetyltrans-

ferases (20, 21). For this reason we used strains that express temperature-sensitive alleles of *ESA1* and *CTF7* (supplemental Tables 1 and 2). The results presented in Fig. 4 show that all the HAT deletion strains and the cells harboring mutant alleles of *ESA1* and *CTF7* retained the H3-K56 acetylation. This suggests either that an as yet unidentified HAT exists or that multiple HATs can acetylate H3-K56.

The identification and genetic characterization of H3-K56 acetylation suggests physiological roles for this histone modification in *S. cerevisiae*. Reversal of the charge at this position (H3-K56E) is lethal (Fig. 2A). This indicates that lysine 56 plays a pivotal role in chromatin structure. The fact that this residue is acetylated underscores the notion that histone core domain residues have biological functions that extend beyond a simple structural role and contribute to regulate chromatin remodeling (22).

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